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ORIGINAL ARTICLE

Loss of *Cntnap2* Causes Axonal Excitability Deficits, Developmental Delay in Cortical Myelination, and Abnormal Stereotyped Motor Behavior

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Abstract

Contactin-associated protein-like 2 (Caspr2) is found at the nodes of Ranvier and has been associated with physiological properties of white matter conductivity. Genetic variation in *CNTNAP2*, the gene encoding Caspr2, has been linked to several neurodevelopmental conditions, yet pathophysiological effects of *CNTNAP2* mutations on axonal physiology and brain myelination are unknown. Here, we have investigated mouse mutants for *Cntnap2* and found profound deficiencies in the clustering of Kv1-family potassium channels in the juxtaparanodes of brain myelinated axons. These deficits are associated with a change in the waveform of axonal action potentials and increases in postsynaptic excitatory responses. We also observed that the normal process of myelination is delayed in *Cntnap2* mutant mice. This later phenotype is a likely modulator of the developmental expressivity of the stereotyped motor behaviors that characterize *Cntnap2* mutant mice. Altogether, our results reveal a mechanism linked to white matter conductivity through which mutation of *CNTNAP2* may affect neurodevelopmental outcomes.

Key words: axonal action potentials, Caspr2, GABAergic interneurons, Kv1-family potassium channels, myelin

Human genetic studies have revealed significant overlaps in genetic variation contributing to multiple neuropsychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics et al.

2013; Guilmatre et al. 2014; McCarthy et al. 2014). One of such examples is variation in *CNTNAP2*, a member of the neurexin superfamily that has been linked to several neurodevelopmental

disorders (Rodenäs-Cuadrado et al. 2014; Poot 2015). In particular, genetic variation in the *CNTNAP2* locus has been associated with childhood apraxia of speech and language impairments, intellectual disability, autism spectrum disorder (ASD), epilepsy, and schizophrenia (Strauss et al. 2006; Friedman et al. 2008; Worthey et al. 2013; Centanni et al. 2015). Although it has been shown that different mutations in the same gene may cause functionally distinct phenotypes (Zhou et al. 2016), the association of a single gene to neurodevelopmental disorders may also be indicative of common pathophysiology (De Rubeis et al. 2014; Fromer et al. 2014). In the case of *CNTNAP2*, the biological mechanisms underlying the contribution of this gene to the developmental trajectory of neuropsychiatric disorders remain unclear (Scott-Van Zeeland et al. 2010; Dennis et al. 2011).

Animal model studies have so far mostly focused on analyzing the consequences of disrupting *Caspr2* function on the balance between excitatory and inhibitory circuits. For example, it has been reported that deficits in the distribution of inhibitory GABAergic neurons may underlie the behavioral alterations found in both mouse and zebrafish *Cntnap2* mutants (Peñagarikano et al. 2011; Hoffman et al. 2016). In mice, loss of cortical GABAergic interneurons has been associated with defects in neural synchronization (Peñagarikano et al. 2011), which reinforces the view that disruption of the excitatory/inhibitory balance might be at the core of the behavioral deficits observed in *Cntnap2* mutants (Peñagarikano et al. 2011; Anderson et al. 2012; Gdalyahu et al. 2015; Jurgensen and Castillo 2015; Varea et al. 2015). In addition, it has been suggested that *Cntnap2* may play a role in synapse development and function (Anderson et al. 2012; Gdalyahu et al. 2015; Varea et al. 2015).

Somehow surprisingly, the most established role for *CNTNAP2* has not yet been considered in the context of neurodevelopmental disorders. *CNTNAP2* encodes contactin-associated protein-like 2 (*Caspr2*), a cell–cell adhesion molecule widely expressed throughout the brain (Gordon et al. 2016) that localizes to the juxtaparanodal region adjacent to the nodes of Ranvier in myelinated axons, where it mediates the interaction between myelinating glia and the axonal membrane (Poliak et al. 2001; Traka et al. 2003). *Caspr2* is required for the clustering of Kv1-family potassium channels at this precise subcellular location (Poliak et al. 1999, 2003; Traka et al. 2003), which are important to stabilize the conduction of axon potentials (Zhou et al. 1998; Vabnick et al. 1999). *Caspr2* is also expressed in the axon initial segment (Inda et al. 2006), but, in contrast to the juxtaparanodes, its function seems dispensable for the clustering of potassium channels in this subcellular region (Ogawa et al. 2008). Based on these observations, it remains to be established whether *Cntnap2* disruption may cause neurodevelopmental deficits through disruption of axonal action potential dynamics or aberrant brain myelination. Their study is of importance to gain understanding of reduced white matter integrity and conductivity implicated in neurodevelopmental disorders (Visser et al. 2012; Rane et al. 2015; Wolff et al. 2015; Fingher et al. 2017).

Here, we investigated axonal physiological properties and postnatal development of myelination in comparison to the emergence of neurophysiological, cognitive, neurological, and behavioral deficits in *Cntnap2* mutant mice. Our results suggest that defects in the propagation of action potentials along myelinated axons contribute to the functional deficits observed in the absence of *Caspr2*. These results reveal a previously unanticipated role for myelination in this process and provide a plausible explanation for the developmental trajectory of the predominantly motor behavioral abnormalities observed in *Cntnap2* mutants. Our findings establish a pervasive mechanism

through which *CNTNAP2* mutations may predispose to a spectrum of neurodevelopmental conditions.

Materials and Methods

Animals

This study was performed in strict accordance with Spanish, British, Dutch, and European Union regulations. Mice were weaned at postnatal (P) day 21, ear punched for genotyping and identification, and socially housed with littermates in groups of 2–5 mice per cage. Mice carrying loss-of-function *Cntnap2* alleles (Poliak et al. 2003) (hereafter called *Cntnap2* mutants) were maintained in a C57BL/6J background.

Histology

Postnatal mice were perfused transcardially with 4% PFA in PBS, and the dissected brains were fixed for 2 h at 4 °C in the same solution. Brains were sectioned at 60 μ m on a vibratome or 40 μ m on a freezing microtome, and free-floating coronal sections were then subsequently processed for immunohistochemistry as previously described (Pla et al. 2006). The following primary antibodies were used: mouse anti-Ankyrin G (1:500, NeuroMab 75-146), rabbit anti-Calretinin (1:1000, Swant 7697), rabbit anti-*Caspr* (1:500), mouse anti-*Caspr* (1:300, NeuroMab 75-001), mouse anti-*Caspr2* (1:400), rat anti-*Ctip2* (1:500, Abcam ab18465), rabbit anti-*Cux1* (1:100, Santa-Cruz CDP-M222), chicken anti-GFP (1:1000, Aves Labs GFP-1020), mouse anti-Kv1.2 potassium channel subunit (1:400, NeuroMab 73-008), mouse anti-Kv1.2 potassium channel subunit (a kind gift from M. N. Rasband), mouse anti-Myelin basic protein (1:500, Merck Millipore MAB384), rabbit anti-Parvalbumin (1:1000, Swant PV27), rabbit anti-Sodium channel (1:300, Sigma-Aldrich S6936), guinea pig anti-*Sox10* (a kind gift from M. Wegner), and rat anti-Somatostatin (1:200, Millipore MAB354).

Electrophysiology

Juvenile (3–4 weeks), adolescent (6–9 weeks), and adult (10–12 weeks) mice were used to prepare acute brain slices. Juvenile animals were anesthetized with pentobarbital and transcardially perfused with cold 95% O₂ + 5% CO₂ sucrose artificial cerebrospinal fluid (aCSF). Coronal slices (350 μ m) were cut using a Leica vibratome (Leica VT 1200 S). Then, they were stored at room temperature for at least 1 h in a submerged holding chamber with 95% O₂/5% CO₂ recording aCSF. For adult animals, we prepared slices (300 μ m) as described before (Ting et al. 2014) with small modifications. Recordings were performed at 22–24 °C.

For extracellular recordings, stimulation of the corpus callosum was performed with a bipolar stimulating electrode (tungsten wires, 75 μ m tip separation, 2 M Ω , WPI), and voltage pulses of 20–30 μ s were applied each 30 s by ISO STIM 01D (NPI Electronic). Antagonists were applied to the recording aCSF to block the synaptic transmission: NBQX (50 μ M), picrotoxin (100 μ M), AP5 (50 μ M), and CGP52432 (5 μ M). Somatic patch-clamp recordings in whole-cell configuration were made from cortical layer 2/3 pyramidal neurons under visual guidance with infrared-differential interference optics (Olympus U-TLUIR) through a 40x water-immersion objective. Excitatory currents were recorded at a holding potential of –70 mV (close to the chloride equilibrium potential) and inhibitory currents at +10 mV (reversal potential of glutamatergic events). For the recordings of miniature currents, tetrodotoxin (1 μ M; Alomone Laboratories) was applied to the extracellular solution. For the

intracellular recordings of evoked currents in layer 2/3 pyramidal neurons, stimulation of the corpus callosum was performed with a bipolar stimulating electrode (tungsten wires, 75 μ m tip separation, 2 M Ω , WPI) positioned under visual control on the callosal tract as described earlier (Kumar and Huguenard 2001) while the membrane potential was held at -70 mV. To exclusively study excitatory responses and to reduce polysynaptic signals, picrotoxin (100 μ M) was included in the recording aCSF to block all GABA_A-receptor-mediated responses.

Image Analysis and Quantification

For the quantification of cell distributions, each animal is considered a biological replication (n). For each animal, about 10–12 sections or imaging field were imaged and treated as technical replicates within the somatosensory cortex and imaged with appropriate excitation and emission requirement based on the staining used. All images were analyzed with customized software written in MATLAB (Mathworks). Layers were defined following nuclear staining.

Behavior

A suite of behavioral paradigms was used to test the developmental onset of abnormalities in male *Cntnap2* mutant mice (Supplementary Fig. 1). All mice were bred and housed under a 12-h light–dark cycle (lights on from 19:00 to 07:00). Before each behavioral experiment, animals were transferred to the test room and habituated for at least 1 h prior to testing. The same sets of mice were tested longitudinally, from early adolescence until adulthood. Details of the behavioral paradigms can be found in the supplementary methods.

Statistical Analyses

Statistical analysis was carried out with IBM SPSS Statistics. P -values below 0.05 were considered statistically significant. Data are presented as mean and standard error of mean (SEM) throughout the article (Supplementary Table 1). Individual trial differences in behavior were determined using one-way ANOVA to test genotype effects. For repeated measurements, a repeated measures ANOVA was performed with “time” as within-subjects factor and “genotype” as between-subjects factor. In case of a significant P value, *post-hoc* comparisons were performed using one-way ANOVA to determine individual time point effects. The involuntary movements and SHIRPA scores were not normally distributed and therefore compared using the general linear model. Normality and variance tests were first applied to all experimental data. When data followed a normal distribution, paired comparisons were analyzed with t -test, while multiple comparisons were analyzed using either ANOVA with *post-hoc* Bonferroni correction (equal variances) or the Welch test with *post-hoc* Games–Howell (different variances). A δ^2 -test was applied to analyze the distribution of cells in layers.

Results

Disrupted Clustering of Potassium Channels in the Brain of *Cntnap2* Mutant Mice

Caspr2 has been previously shown to be required for the normal clustering of potassium channels in the juxtaparanodal region of the nodes of Ranvier in myelinated peripheral axons (Poliak et al. 2003). We wondered whether a similar defect was present in long-range cortical axons, which are also densely

myelinated (Tomassy et al. 2014). In 8-week-old mice, Caspr2 expression is abundant in the corpus callosum, which primarily comprises interhemispheric axons from pyramidal cells located in superficial layers of the cortex (Fig. 1A,B). At the subcellular level, Caspr2 is found in the juxtaparanodal region of the nodes of Ranvier (Fig. 1C,D,E), immediately flanking the paranodal junction. As reported earlier in peripheral nervous system axons (Poliak et al. 2003), we observed that Caspr and sodium channels are properly located at the paranodal junction and the nodes, respectively, in *Cntnap2* mutants (Fig. 1C,D–G). In contrast, clustering of Kv1.2 channels is severely disrupted in cortical myelinated axons in the corpus callosum of *Cntnap2* mutant mice compared with controls (Fig. 1H–K). We observed a prominent reduction in the number of nodes containing symmetric Kv1.2 clusters and a parallel increase in the frequency of nodes with asymmetric clusters or with complete absence of Kv1.2 clusters (Fig. 1L). Similar defects were observed in other regions of the telencephalon, including the internal capsule traversing the striatum and the external capsule (data not shown). Altogether, these results revealed that the organization of potassium channels in brain myelinated axons is severely disrupted in *Cntnap2* mutant mice.

Abnormal Axonal Action Potential Waveform in *Cntnap2* Mutant Mice

The functional consequences of the abnormal clustering of potassium channels in the juxtaparanodal region of the nodes of Ranvier are poorly understood. Previous studies in both optic and sciatic nerves of adult *Cntnap2* mutant mice revealed no apparent changes in nerve conduction (Poliak et al. 2003). However, the absence of clustered potassium channels at the nodes of Ranvier is expected to have an impact in action potential waveform and axonal excitability (Vabnick et al. 1999; Vivekananda et al. 2017). To test this hypothesis, we investigated global axonal electrical activity in acute cortical slices from 8-week-old mice. To this end, we first stimulated the corpus callosum in one hemisphere and recorded the fiber volleys (FV) evoked from the contralateral hemisphere (Fig. 2A). We observed that FV amplitudes are significantly reduced in *Cntnap2* mutant mice compared with controls (Fig. 2B,C), indicative of changes in axonal action potential waveform, fiber recruitment, or axonal density. Analysis of the distribution of pyramidal cells in the neocortex of control and *Cntnap2* mutant mice revealed no significant differences (Supplementary Fig. 2A–F). To confirm this observation, we carried out *in utero* electroporation experiments in which we labeled callosal layer 2/3 neurons with a plasmid encoding *Gfp* (Supplementary Fig. 2G). These experiments confirmed that pyramidal cell migration is not altered in the neocortex of *Cntnap2* mutants. In addition, we observed a similar organization of callosal axons in both genotypes (Supplementary Fig. 2H–K).

The previous results suggested that the differences in FV amplitudes between control and *Cntnap2* mutant mice are likely due to changes in the axonal action potential waveform and/or changes in fiber recruitment. To discriminate between these possibilities, we recorded single-action potentials in loose-patch configuration by drawing individual axons from the corpus callosum into suction electrodes. All-or-none action potentials were recorded after minimal stimulation from the contralateral corpus callosum. In this configuration, axonal deflections closely follow the first derivative of the action potential, where the peak corresponds to the maximal rise slope due to the opening of voltage-gated Na⁺ conductances

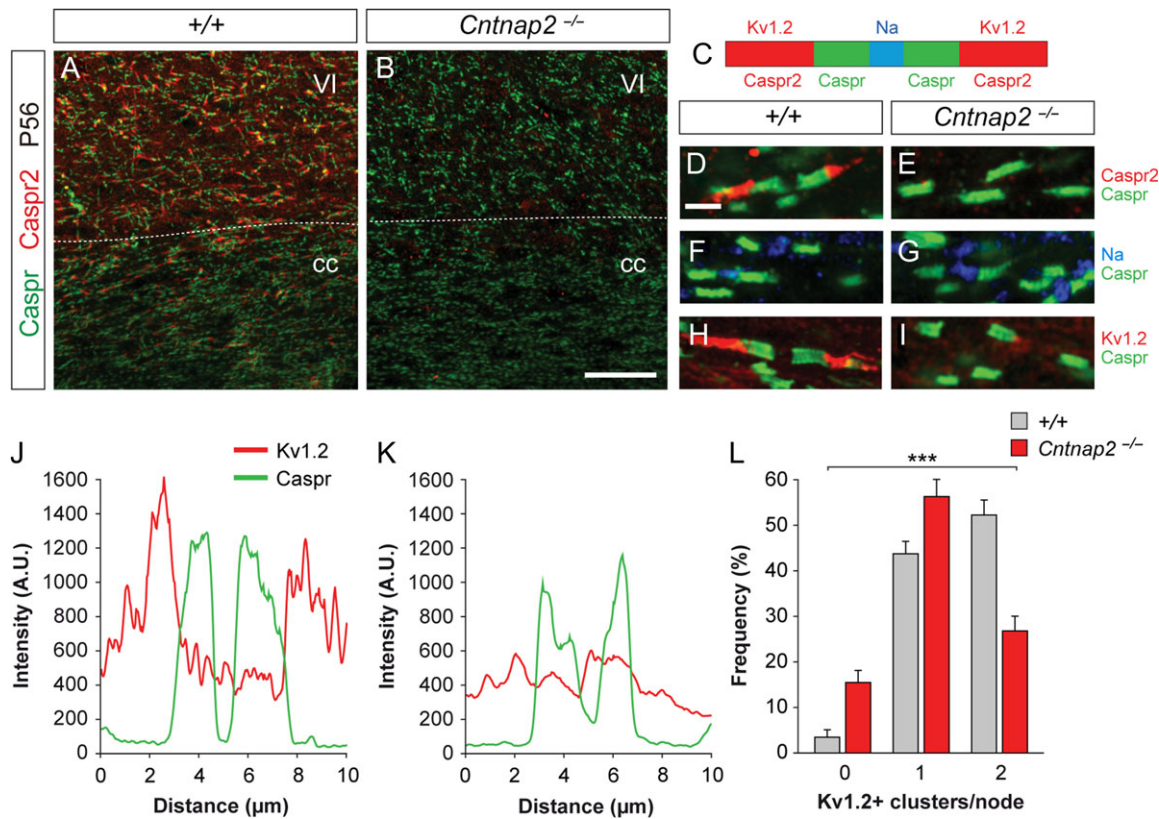


Figure 1. Abnormal clustering of Kv1.2 channels at the nodes of Ranvier in the telencephalon of *Cntnap2* mutant mice. (A, B) Caspr and Caspr2 expression in the corpus callosum (cc) and layer 6 of the neocortex in 8-week-old control (A) and *Cntnap2* mutant (B) mice. (C) Schematic illustrating the normal distribution of proteins at the node (blue), paranodes (green), and juxtaparanodes (red). (D–I) High-magnification images illustrating the expression of Caspr (green; D–I), Caspr2 (red; D, E), Na⁺ channels (blue; F, G), and Kv1.2 (red; H, I) channels at the node of Ranvier in corpus callosum axons from control (D, F, and H) and *Cntnap2* mutant (E, G, I) mice. (J, K) Representative traces depicting relative levels of expression of Caspr and Kv1.2 at the paranode and juxtaparanode in control (J) and *Cntnap2* mutant (K) mice. These traces were used to determine the number of Caspr and Kv1.2 clusters present in each node. (L) Quantification of the relative frequency of nodes of Ranvier containing zero, 1, or 2 Kv1.2 clusters in control and *Cntnap2* mutant mice; $n = 263$ and 201 nodes from 3 control and 3 *Cntnap2* mutant mice, respectively; χ^2 -test, *** $P = 0.001$. Histograms show average \pm SEM. Scale bars equal 200 μ m (A, B) and 5 μ m (D–I).

and the anti-peak corresponds to the maximal decay slope due to the opening of voltage-gated K⁺ conductances (Henze et al. 2000; Scott et al. 2014). We observed that anti-peak amplitudes are substantially reduced in 8-week-old *Cntnap2* mutant mice compared with controls (Fig. 2D,E), a phenotype that is already apparent at 3 weeks of age (Fig. 2F,G). These results suggested that the improper distribution of Kv1-family channels in the axon slows down the repolarization phase of the action potential, thereby modifying the spike waveform in long-range myelinated axons.

Abnormal Excitatory Synaptic Transmission in the Neocortex of *Cntnap2* Mutant Mice

Changes in axonal action potential shape greatly alter neurotransmitter release (Sabatini and Regehr 1997), and these effects can be even passively transmitted at distal synaptic release sites (Alle and Geiger 2006; Shu et al. 2006). To evaluate the potential impact of the biophysical changes in action potential waveform observed in myelinated axons lacking Caspr2, we first recorded spontaneous excitatory postsynaptic currents (sEPSCs) from layer 2/3 pyramidal neurons in 8-week-old mice. We observed that while the frequency of excitatory events recorded in these cells was similar for both genotypes (Fig. 3A,C), the mean amplitude of sEPSCs was significantly

higher in pyramidal cells from *Cntnap2* mutant mice (Fig. 3A,D). Since this phenotype could be caused by a change in the number of excitatory synapses, we measured miniature events with whole-cell recordings. Analysis of miniature excitatory postsynaptic currents showed no significant differences in the frequency and amplitude of synaptic events in pyramidal cells of *Cntnap2* mutant mice compared with controls (Supplementary Fig. 3A,C,D). Consequently, the abnormal rise in sEPSCs amplitude is likely linked to increased neurotransmitter release due to wider axonal action potentials in the myelinated presynaptic excitatory neurons.

Next we recorded evoked excitatory responses (eEPSCs) from layer 2/3 pyramidal cells following stimulation in the corpus callosum (Fig. 3G). Following the first stimulus, we observed a significant increase in the amplitude of eEPSCs in pyramidal cells from *Cntnap2* mutants compared with controls (Fig. 3H,I). Moreover, we found a prominent decrease in the PPR when paired stimuli were applied as part of the stimulation protocol (Fig. 3J), indicating a reduced probability of release from these synapses in *Cntnap2* mutants during the second stimulus. These results strongly supported the notion that excitatory synaptic transmission is abnormally enhanced in the absence of Caspr2. Finally, to confirm that this phenotype is caused by the abnormally high release of excitatory terminals in *Cntnap2* mutant mice, we performed another series of experiments in

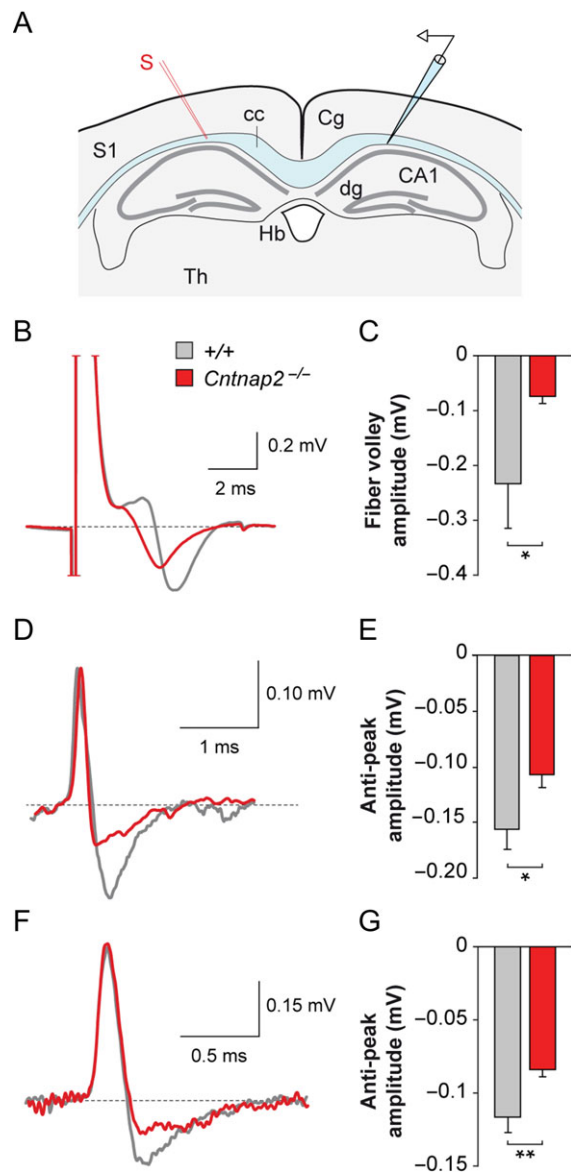


Figure 2. Action potential repolarization in cortical axons is altered in *Cntnap2* mutant mice. (A) Schematic of the experimental design. (B) Representative FV traces from 3-week-old control and *Cntnap2* mutant mice. Traces are the mean of 10 sweeps. The traces were subtracted after the application of tetrodotoxin to reduce the stimulating artifact. (C) Quantification of FV amplitudes; $n = 8$ FV from 8 control mice and 10 FV from 9 *Cntnap2* mutant mice; t -test, $^{*}P < 0.05$. (D) Representative traces of loose-patch single-axon potentials recorded in the corpus callosum of 8-week-old control and *Cntnap2* mutant mice. (E) Quantification of anti-peak amplitudes; $n = 39$ axons from control mice and 39 axons from *Cntnap2* mutant mice at 8 weeks; t -test, $^{*}P < 0.05$. (F) Representative traces of loose-patch single-axon potentials recorded in the corpus callosum of 3-week-old control and *Cntnap2* mutant mice. (G) Quantification of anti-peak amplitudes; $n = 41$ axons from control mice and 44 axons from *Cntnap2* mutant mice; t -test, $^{**}P < 0.01$. Histograms show average \pm SEM. CA1, CA1 region of the hippocampus; cc, corpus callosum; Cg, cingulate cortex; dg, dentate gyrus; Hb, habenula; S, stimulating electrode; S1, primary somatosensory cortex; Th, thalamus.

which the concentration of extracellular calcium was reduced to 1 mM to decrease the probability of neurotransmitter release. We found that PPR differences between both genotypes are neutralized under these conditions (Fig. 3K,L), which reinforced the idea that an increase in release probability at

excitatory neurons causes the abnormally high amplitude of excitatory responses observed in pyramidal cells from *Cntnap2* mutants.

Previous studies have reported deficits in the number of cortical interneurons in early postnatal *Cntnap2* mutant mice (Peñagarikano et al. 2011). To test the possible involvement of inhibition in the abnormally high amplitude of excitatory responses observed in *Cntnap2* mutant mice, we investigated the extent of interneuron deficits in 8-week-old mice. We recorded sIPSCs from layer 2/3 pyramidal neurons in 8-week-old mice and found no changes in either the frequency or amplitude of sIPSCs (Fig. 3B,E,F). Consistent with this observation, we found no significant differences in the density and laminar distribution of cortical interneurons in the somatosensory cortex of *Cntnap2* mutant mice compared with controls (Fig. 4). Similarly, the distribution of hippocampal interneurons was comparable between both genotypes (data not shown). To extend these observations, we analyzed miniature inhibitory postsynaptic currents with whole-cell recordings from pyramidal cells and found no significant differences in the frequency and amplitude of synaptic events in pyramidal cells of *Cntnap2* mutant mice compared with controls (Supplementary Fig. 3B,E,F). Altogether, these observations revealed that inhibitory connectivity is normal in the neocortex of young adult *Cntnap2* mutant mice.

Delayed Gray Matter Myelination in *Cntnap2* Mutant Mice

The previous results suggested that defects in excitatory neurotransmission are characteristic in *Cntnap2* mutant mice. We reasoned that a critical factor for the expressivity of the axonal action potential waveform phenotype observed in *Cntnap2* mutant mice is myelination, since only axons that are fully myelinated would maximally benefit from the clustered organization of potassium channels at the nodes of Ranvier. Interestingly, we found a significant reduction in gray matter myelination of the neocortex in juvenile *Cntnap2* mutant mice (Fig. 5A–C, F–H). In particular, while myelinated fibers have a comparable density in the corpus callosum and infragranular layers of the cortex in controls and *Cntnap2* mutants, the density of myelinated axons in superficial layers of the neocortex was decreased in the absence of *Caspr2* (Fig. 5C,E,H). Consistent with this observation, we found a significant reduction in the density of Sox10+ cells in the neocortex of 3-week-old *Cntnap2* mutants compared with control mice (Fig. 5D,I,J).

The reduced myelination in the neocortex of juvenile *Cntnap2* mutant mice was also obvious in electrophysiological recordings measuring the speed of propagation of axon potentials. In these experiments, we stimulated the corpus callosum and recorded the local field potential (LFP) at progressively more distant sites, within the corpus callosum and also in the gray matter, while synaptic transmission was completely blocked with specific drugs (Fig. 5K). In these conditions, the LFP reflected mostly the action potentials propagating through the stimulated axons (Swadlow 1974). As suggested by the myelin staining, we found no differences in the speed of the action potentials propagating within the corpus callosum (up to 3000 μ m from the stimulating electrode; Fig. 5L,M) between both genotypes. However, the speed of propagation was on average significantly slower in *Cntnap2* mutants than in control mice when the recordings were made within the gray matter (3000–6000 μ m from the stimulating electrode; Fig. 5L,M), consistent with the reduction in the density of myelinated fibers

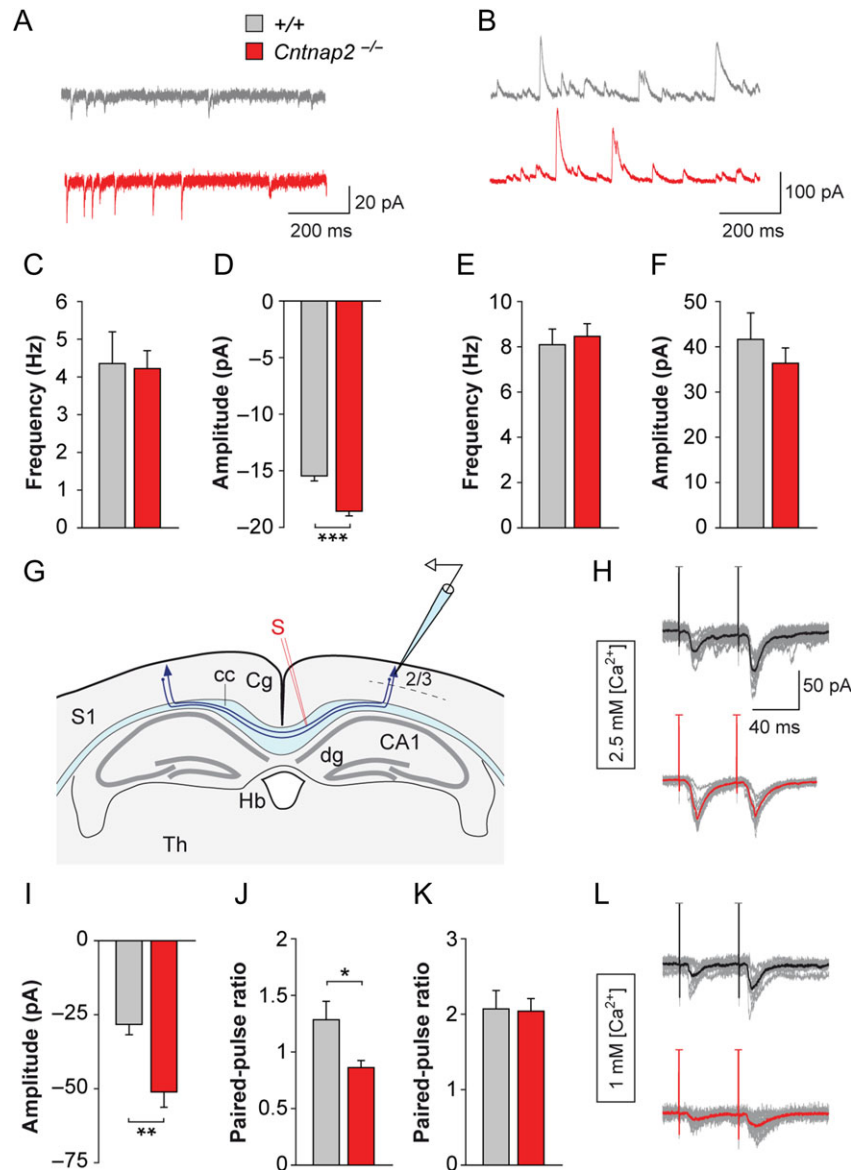


Figure 3. Abnormal excitatory synaptic transmission in the neocortex of *Cntnap2* mutant mice. (A, B) Representative traces of sEPSCs and spontaneous inhibitory post-synaptic currents (sIPSCs) recorded from layer 2/3 pyramidal neurons in control and *Cntnap2* mutant mice. (C, D) Quantification of sEPSCs frequencies (C) and amplitudes (D) in pyramidal cells; $n = 13$ and 18 cells in control and *Cntnap2* mutant mice, respectively; t -test, $P = 0.56$ (C) and $*P < 0.001$ (D). (E, F) Quantification of sIPSCs frequencies (E) and amplitudes (F) in pyramidal cells; $n = 13$ and 18 cells in control and *Cntnap2* mutant mice, respectively; t -test, $P = 0.79$ (E) and $P = 0.46$ (F). (G) Schematic of the experimental design. (H, I) Representative traces of eEPSCs recorded from layer 2/3 pyramidal neurons following paired pulses in control and *Cntnap2* mutant mice with 2.5 mM (H) and 1 mM (I) extracellular calcium. (J) Quantification of EPSCs amplitudes evoked in control and *Cntnap2* mutant mice; $n = 18$ and 17 cells in control and *Cntnap2* mutant mice, respectively; t -test, $*P < 0.01$. (K, L) Quantification of paired-pulse ratios (PPR) in control and *Cntnap2* mutant mice with 2.5 mM (K) and 1 mM (L) extracellular calcium; $n = 14$ and 13 cells in control and *Cntnap2* mutant mice, respectively; t -test, $*P < 0.05$ (K) and $P = 0.87$ (L). Histograms show average \pm SEM. CA1, CA1 region of the hippocampus; cc, corpus callosum; Cg, cingulate cortex; dg, dentate gyrus; Hb, habenula; S, stimulating electrode; S1, primary somatosensory cortex; Th, thalamus; 2/3, pyramidal cell in cortical layer 2/3.

observed in these mice. In particular, axons with fast propagation speeds (i.e., highly myelinated) were nearly absent from the recordings in *Cntnap2* mutants.

To distinguish whether the defects in myelination were transitory or permanent, we repeated these analyses in a cohort of 8-week-old mice. We observed a recovery in the myelination of the neocortex in *Cntnap2* mutants: the density of myelinated fibers, number of Sox10+ cells, and propagation speeds were comparable between both genotypes (Supplementary Fig. 4). These results suggested that myelination is only transiently compromised in the neocortex of *Cntnap2* mutant mice.

Developmental Onset of Repetitive Behaviors *Cntnap2* Mutant Mice

Given the wide implication of *Caspr2* disruption in developmental disorders, we investigated the behavior of *Cntnap2* mutant mice using longitudinal assessment across postnatal developmental stages (Supplementary Fig. 1). At 4 weeks, we found no significant differences in the assessment of *Cntnap2* mutant mice compared with littermate wild-type controls (Fig. 6A–C). From 6 weeks of age, significant behavioral abnormalities were found in *Cntnap2* mutants compared with control mice. Exposed to a novel

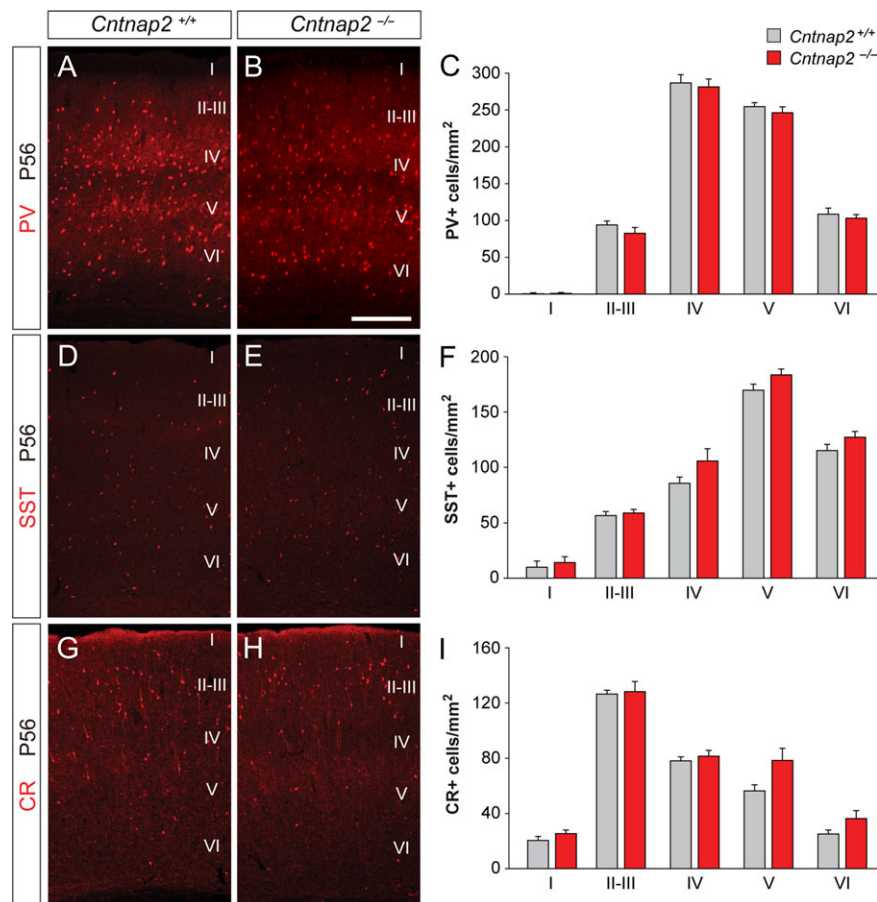


Figure 4. Normal distribution of cortical interneurons in the neocortex of adult *Cntnap2* mutant mice. (A, B, D, E, G, H) Distribution of PV (A, B), SST+ (D, E), and CR+ (G, H) interneurons in the somatosensory cortex of control (A, D, G) and *Cntnap2* mutant (B, E, H) mice. (C, F, I) Laminar distribution of PV+ (C), SST+ (F), and CR+ (I) interneurons; $n = 4$ control and *Cntnap2* mutant mice, 2-way ANOVA, $P = 0.98$ (C), $P = 0.88$ (F), and $P = 0.78$ (I). Scale bar equals 200 μm .

empty cage, we observed increased repetitive behaviors in *Cntnap2* mutant mice that persisted into adulthood. These include increased grooming behavior and 2 previously unrecognized stereotyped motor phenotypes. One novel behavior was found in the grooming sequence; distinct episodes were identified when the ears were groomed alternately and repeatedly. We classified this behavior as rubbing. A second novel behavioral phenotype was identified as sudden non-rhythmic jerk-like movements of the whole body or body parts, further referred to as involuntary movements or tic-like behavior (Fig. 6A–D). In contrast, no differences were observed in rearing behavior (Supplementary Fig. 5A) or motor activity levels (data not shown) across the different developmental stages.

To establish whether these stereotyped motor behaviors might be due to reduced sensorimotor co-ordination, we examined motor balance and sensorimotor competence using the accelerating rotarod. We found no differences in performance between controls and littermate *Cntnap2* mutant mice at any of the ages examined (Supplementary Fig. 5B). In addition, *Cntnap2* mutants exhibited no alteration in reflexes, muscle strength, and sensory responses across the developmental stages (Supplementary Table 2), while a modest reduction in body and brain weights compared with controls was found (Supplementary Fig. 6A,B).

At adult age, we tested a multi-trial compound set-shifting paradigm and found no evidence that the repetitive behaviors in *Cntnap2* mutants were associated with cognitive inflexibility

(Supplementary Fig. 7A). Subsequently, we used the home cage for unbiased monitoring of basic behavioral readouts. Assessment of feeding behavior indicated that *Cntnap2* mutant mice have similar levels of food intake, but duration of intake was reduced (Supplementary Fig. 6C,D). *Cntnap2* mutant mice displayed locomotor hyperactivity mainly when exposed to the home cage for the first time (Fig. 6F) and during the light phase, the habitual sleep phase for this nocturnal species (Fig. 6E and Supplementary Fig. 5C). In contrast, overall distance moved in the home cage was similar between control and *Cntnap2* mutant mice during the dark phase, the habitual activity phase (Fig. 6E and Supplementary Fig. 5C). Increased motor activity levels were also found when exposed to the elevated plus maze (Supplementary Fig. 5D). This activity in *Cntnap2* mutant mice compared with controls was restricted to the open arms of the maze (Supplementary Fig. 5E). This finding could suggest reduced anxiety-like behaviors in the absence of *Cntnap2*, which was contradicted by similar levels of inner and outer zone activity in the open field test (Supplementary Fig. 5F,G).

Finally, we tested the social behavioral domain and found that *Cntnap2* mutant mice showed similar levels of social interaction with genotype-matched conspecifics in a juvenile P21 social behavior test compared with controls. During this test, increased rubbing in the *Cntnap2* mutant mice was already evident (Supplementary Fig. 7B). At adult age, sociability in the standard 3-chamber paradigm task, defined as spending more

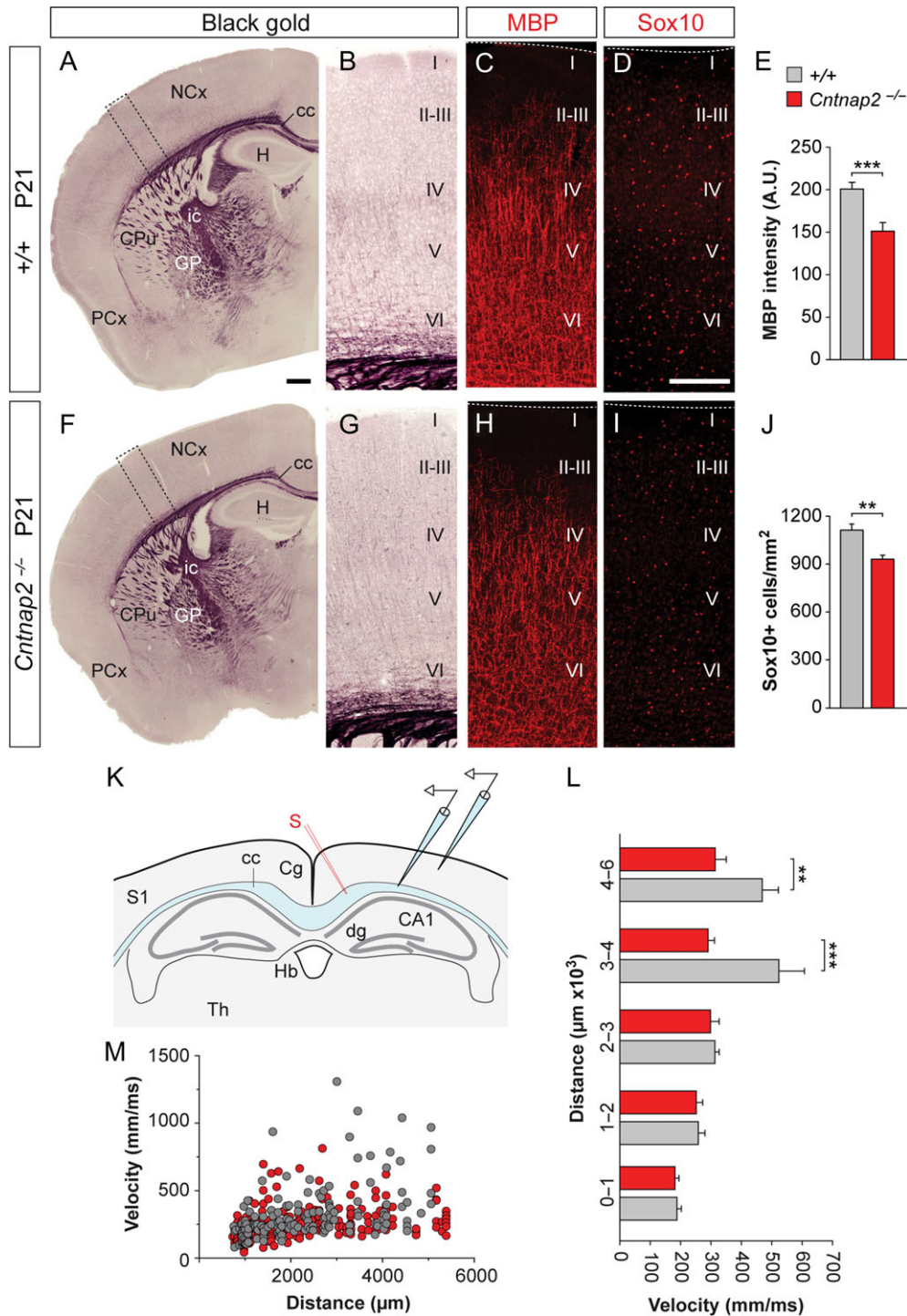


Figure 5. Delayed myelination of cortical gray matter in *Cntnap2* mutant mice. (A–D, F–I) Histological staining of myelin by Black gold (A, B, F, G) and immunohistochemistry for MBP (C, H) and Sox10 (D, I) in the neocortex of 3-week-old control (A–D) and *Cntnap2* mutant (F–I) mice. (E) Quantification of MBP intensity in the somatosensory cortex; $n = 4$ control and 4 *Cntnap2* mutant mice, t -test, *** $P < 0.001$. (J) Quantification of the density of Sox10+ cells in the somatosensory cortex; $n = 4$ control and 4 *Cntnap2* mutant mice, t -test, ** $P < 0.01$. (K) Schematic of the experimental design. (L) Quantification of axonal conduction speeds as a function of distance. Bins under 3000 μm correspond to recordings within the corpus callosum, while bins over 3000 μm correspond to recordings within the cortical gray matter; $n = 152$ axons from control mice and 187 axons from *Cntnap2* mutant mice, 2-way ANOVA, ** $P < 0.01$, *** $P < 0.001$. (M) Distribution of axonal conduction speed for individual axons at different distances from the stimulation electrode. Histograms show average \pm SEM. CA1, CA1 region of the hippocampus; cc, corpus callosum; Cg, cingulate cortex; dg, dentate gyrus; Hb, habenula; S, stimulating electrode; S1, primary somatosensory cortex; Th, thalamus; 2/3, pyramidal cell in cortical layer 2/3. Scale bar equals 200 μm .

time with the novel mouse than with the novel object, were similar for control and *Cntnap2* mutant mice (Supplementary Fig. 7C). Social recognition memory in a direct social

interaction test was also unmarked in both groups. Mice from both genotypes spent more time sniffing a novel than a familiar mouse (Supplementary Fig. 7D). These data suggest that

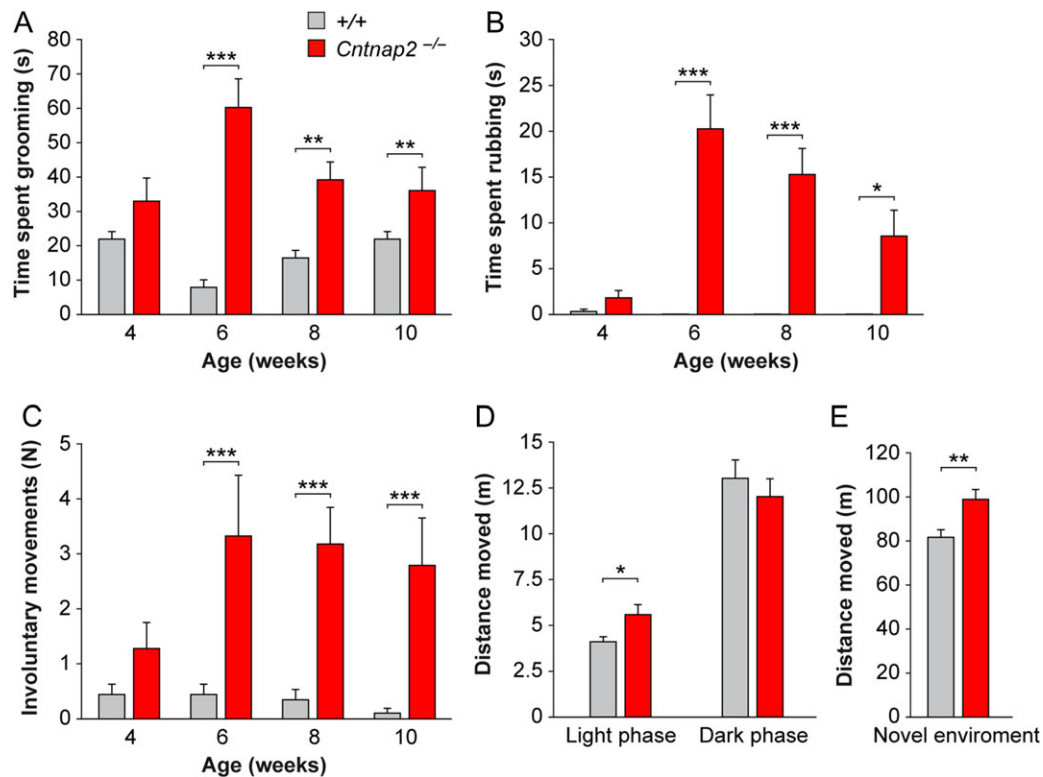


Figure 6. Developmental onset of motor abnormalities and repetitive behaviors in *Cntnap2* mutant mice. (A–C) Quantification of time spent grooming (A) or rubbing (B), and total amount of involuntary movements (C). (D) Quantification of distance moved in light and dark phase. (E) Quantification of activity for 1 h in a novel environment. (G) Tracking of novel exploration for control and *Cntnap2* mutant mice in home cage. Histograms show average ± SEM. *n* = 11–14 control and 10–12 *Cntnap2* mutant mice; RM-ANOVA, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Cntnap2 mutant mice do not have aberrant development of social interaction behavior.

Discussion

Our results indicate that loss of *Caspr2* modifies the action potential waveform in central myelinated axons and causes an abnormal increase in neurotransmitter release. In the neocortex, for instance, loss of *Caspr2* leads to increased postsynaptic excitatory responses in pyramidal cells. Our data suggest that the axonal biophysical changes observed in *Cntnap2* mutants are likely due to the abnormal clustering of Kv1-family potassium channels in the juxtaparanodes region of the nodes of Ranvier, a phenotype whose expressivity is linked to myelination. Consequently, relatively subtle defects in functional connectivity are likely widespread among central myelinated axons in *Cntnap2* mutants and may contribute to the developmental trajectory of behavioral deficits here established for *Cntnap2* mutants. Our results reveal a pervasive mechanism through which *CNTNAP2* mutations may predispose to neurodevelopmental conditions in humans.

Abnormal Neurotransmitter Release in *Cntnap2* Mutant Myelinated Axons

Synaptic transmission can be modulated by electrotonic propagation of subthreshold membrane depolarization along axons (Alle and Geiger 2006; Shu et al. 2006). Previous studies have shown that depolarization-mediated inactivation of axonal Kv1-family potassium channels contribute to this form of “analog”

signaling by broadening action potentials (Kole et al. 2007; Shu et al. 2007). Moreover, recent experiments using ion conductance microscopy have demonstrated that pharmacological inhibition and genetic deletion of Kv1.1 channels broaden presynaptic spikes at intact axonal boutons (Vivekananda et al. 2017). Consistently, we observed that defective clustering of Kv1 channels in myelinated axons of *Cntnap2* mutants modifies the shape of presynaptic action potentials. As previously shown in other central synapses, presynaptic spike broadening leads to a proportional increase in Ca^{2+} influx (Geiger and Jonas 2000; Begum et al. 2016). Consequently, the functional consequence of the defective clustering of Kv1 channels in central myelinated axons is an abnormal increase in neurotransmitter release. In the neocortex, these defects translate into increased excitatory synaptic input onto pyramidal cells, as revealed by the higher amplitude of EPSCs observed in layer 2/3 pyramidal cells. Since the defects found in the clustering of Kv1 channels are likely present in all myelinated neurons, the abnormal increase in synaptic responses is probably present in other brain areas. It is conceivable, for instance, that pyramidal cells in layer 5 may also elicit increased excitatory responses in the striatum and other subcortical targets. Although *Cntnap2* mutants do not display overt seizures, it is possible that the defects in excitatory neurotransmission described here might be related to the abnormal increase in asymptomatic seizure-like spiking events observed in the cortex of *Cntnap2* mutants during EEG recordings (Thomas et al. 2016).

Previous work described a significant reduction in the number of cortical inhibitory neurons as the most likely cause underlying the behavioral defects observed in *Cntnap2* mutant mice (Peñagarikano et al. 2011). Our results, however, suggest that

neocortical inhibitory circuits are grossly normal in developing and adult *Cntnap2* mutants. These results are consistent with the observations that juvenile and young adult *Cntnap2* mutant mice do not exhibit seizures during behavioral testing (Brunner et al. 2015; Thomas et al. 2016). It should be noted, however, that deficits in inhibitory synaptic transmission have been reported in the hippocampus of *Cntnap2* mutant mice (Jurgensen and Castillo 2015). Therefore, it is possible that the functional consequences of disrupting *Caspr2* function may vary among different cortical areas.

Our results also suggest that the laminar distribution of neocortical pyramidal cells is apparently normal in *Cntnap2* mutants. Cortical dysplasia was reported in a previous analysis of *Cntnap2* mutants (Peñagarikano et al. 2011) and in the original description of patients carrying homozygous deletions in the *CNTNAP2* locus (Strauss et al. 2006). It is worth noting, however, that cortical dysplasia is not present in all patients carrying bi-allelic *CNTNAP2* mutations, even with a similar clinical picture (Smogavec et al. 2016). Consequently, cortical dysplasia might not be as common in *CNTNAP2*-related disorders as assumed previously. In addition, we found no obvious defects in the connectivity of layer 2/3 pyramidal cells, in contrast to previous reports (Anderson et al. 2012; Gdalyahu et al. 2015). One possibility is that the loss of *Caspr2* in vivo impacts differentially the connectivity of pyramidal cells in different layers and regions of the neocortex, since the loss of dendritic spines has been reported for layer 5 pyramidal cells (Gdalyahu et al. 2015) and our analysis was restricted to layer 2/3 neurons.

Cortical Myelination Defects in *Cntnap2* Mutant Mice

Myelin plays a critical role enabling neuronal function, and defects in myelination have been linked to multiple neurological and neuropsychiatric disorders (Nave 2010). Our observations indicate that myelination is delayed in the neocortex of *Cntnap2* mutant mice, most probably due to an early deficit in the number of oligodendrocytes that seems to be compensated in the adult cortex. Although the precise mechanisms underlying this phenotype remain to be investigated, it is well established that the proliferation of oligodendrocyte precursor cells depends on the electrical activity of axons (Barres and Raff 1993).

Resting-state fMRI studies in mice suggest that white matter connectivity is normal in adult *Cntnap2* mutant mice (Liska et al. 2017), which is consistent with our histological and electrophysiological observations at 8 weeks. However, the reduced myelination observed in juvenile *Cntnap2* mutant mice is likely responsible for the absence of fast propagation speeds observed at this stage. This phenotype, during a critical developmental window, may influence network dynamics of cortical neurons and perturb the consolidation of long-range functional connectivity (Wang et al. 2008), as previously described in ASD (Vissers et al. 2012; Rane et al. 2015). Recent imaging studies in humans have also reported transient defects in the corpus callosum of young toddlers later diagnosed with ASD (Wolff et al. 2015; Fingher et al. 2017), prior to the onset of behavioral abnormalities.

Expressivity of Neurodevelopmental Phenotypes in *Cntnap2* Mutant Mice

Our experiments revealed defects in the waveform of axonal action potentials in *Cntnap2* mutant mice at 3 weeks of age, prior to the onset of behavioral abnormalities. There are 2 possible, non-exclusive explanations for this divergence. Firstly,

the characteristic organization of channels at the nodes of Ranvier, which likely underlies the changes in the waveform of axonal action potentials observed in *Cntnap2* mutants, is directly linked to the process of myelination. Indeed, channels cluster progressively in their mature location in parallel to the process of myelination (Rasband et al. 1999; Vabnick et al. 1999), and so their impact on axonal physiology increases with age under normal circumstances. Since myelination is delayed in *Cntnap2* mutants, the relatively late onset of behavioral phenotypes may indicate that the consequences of the changes in axonal physiology may only manifest fully when myelination is completed.

Second, clinical research and experimental manipulations in rodents suggest a role for abnormal function of cortico-striatal-thalamo-cortical circuits in repetitive motor behaviors (Saxena et al. 1998; Marsh et al. 2009; Ahmari et al. 2013; Burguiere et al. 2013). The nature of the stereotyped motor behaviors observed in *Cntnap2* mutants reflects inadequate coping and arousal in response to unexpected or novel situations (Turner 1999; Richler et al. 2007; Geurts et al. 2009; Lewis and Kim 2009), which is consistent with a role of cortico-striatal circuits independently of a more complex set of mental operations involved in cognitive flexibility and social interactions (Geurts et al. 2009). Interestingly, repeated – but not acute – optogenetic hyperactivation of cortico-striatal connections over multiple days generates a progressive increase in grooming in mice (Ahmari et al. 2013). Thus, it is conceivable that a sustained increase in postsynaptic responses at cortico-striatal synapses during early postnatal development may lead to the motor stereotypes characteristic of *Cntnap2* mutant mice, although this hypothesis remains to be experimentally tested.

Supplementary Material

Supplementary data are available at *Cerebral Cortex* online.

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